BIOSYNTHESIS OF MITOCHONDRIAL CITRATE SYNTHASE IN *NEUROSPORA CRASSA*

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1. Introduction

The transfer of mitochondrial proteins coded for by nuclear DNA from their site of synthesis into the mitochondria is currently attracting much attention. Early proposals that this transfer occurs by a co-translational mechanism [1] have not been substantiated, rather evidence has been produced which supports a post-translational mechanism involving extramitochondrial precursors [2–6]. The nature of these extramitochondrial precursors has been investigated for a number of proteins [4–12]. In the case of some proteins the initial translation products are larger than the mature protein [6–8,11] while this does not hold for others [4,5,9,10,12]. As no general rule of behaviour has been observed and no general hypothesis advanced it is necessary to investigate the formation of proteins having different mitochondrial locations. In this context we have studied the synthesis of citrate synthase (EC 4.1.3.7), a soluble matrix protein consisting of two identical subunits [13,14]. The data presented indicate that this protein is synthesized as a larger molecular weight precursor. Synthesis of the precursor and its subsequent transfer into the mitochondria are temporally separate in whole cells. Conversion of the precursor to the mature protein is by post-translational cleavage.

2. Materials and methods

Experimental conditions are only described where they differ from the procedures in [2–4,9,10].

Citrate synthase was prepared from purified mitochondria using ATP–Sepharose chromatography as in [14]. Antibodies were raised against the isolated protein as in [2]. Immunoprecipitation was carried out on cell fractions lysed by the addition of Triton X-100 to 1% and KCl to 0.3 M. Immunoglobulin was added at 80 μg/ml and the lysate shaken for 5 min. Sepharose bound protein A (10 mg, swollen in 10 vol. Tris–HCl (pH 7.4), 10 mM) was added and the lysate shaken for a further 5 min. The protein A–Sepharose immunoglobulin complex was centrifuged, washed and dissociated by shaking for 1 h in 10 mM Tris–HCl (pH 6.8), 2.5% SDS, 5% mercaptoethanol at 4°C followed by 5 min at 95°C.

For pulse labelling with sodium [^{35}S]sulphate, cells were depleted of sulphate by transfer to a sulphate-free medium for 2 h. Labelling was by incubating cells at 8°C with sodium [^{35}S]sulphate (10 mCi/l, spec. act. 10–1000 mCi/mol). Cells were quickly chilled, harvested and frozen in liquid nitrogen. The frozen cells were ground in a mortar and extracted with 0.5 M sucrose, 10 mM Tris–HCl, 5 mM NH_4Cl, 1 mM mercaptoethanol, 1 mM phenylmethansulphonylfluoride (pH 7.5). The homogenate was centrifuged at 12 000 × *g* for 12 min and citrate synthase immunoprecipitated from the supernatant.

3. Results

When citrate synthase isolated from *Neurospora crassa* mitochondria was subjected to SDS–polyacrylamide electrophoresis it displayed M_r 45 000 (fig.1). This is similar to the subunit molecular weights re-

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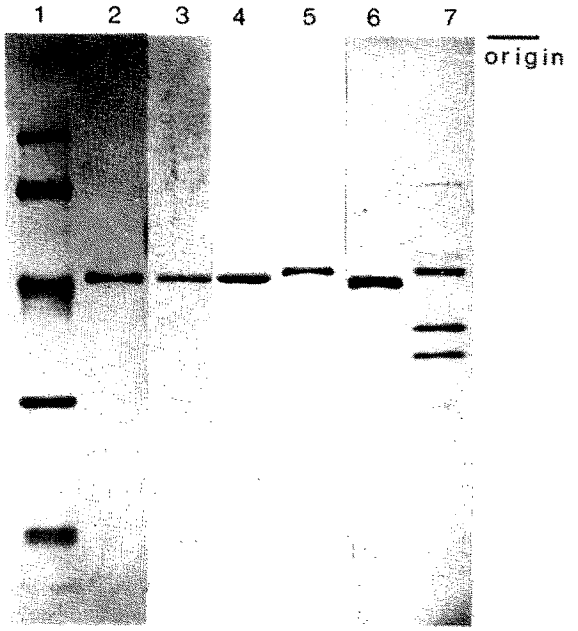
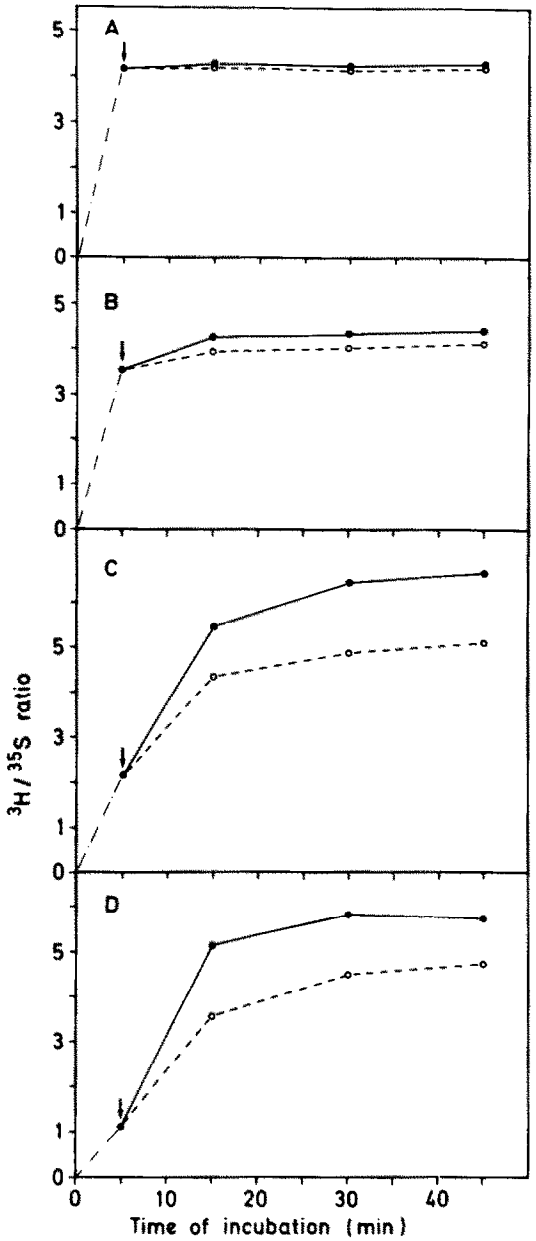


Fig.1. SDS electrophoretic analysis of mature and precursor forms of citrate synthase. (1,2) Protein stain; (3–7) autoradiographs. (1) Protein standards (94 k, 67 k, 43 k, 30 k, 20 k); (2) isolated citrate synthase; (3) citrate synthase immunoprecipitated from purified mitochondria isolated from cells labelled with [^{35}S]sulphate; (4,6) citrate synthase immunoprecipitated from post-mitochondrial supernatant obtained from cells labelled with [^{35}S]sulphate; (5) citrate synthase immunoprecipitated from a homologous cell-free translation system labelled with [^{35}S]methionine; (7) citrate synthase immunoprecipitated from a rabbit reticulocyte lysate programmed with poly(A)RNA and labelled with [^{35}S]methionine.

Fig.2. Pulse chase kinetics of incorporation of leucine into citrate synthase in vivo. Cells grown in the presence of [^{35}S]sulphate were labelled with [^{3}H]leucine at 8°C. One portion of the culture received a chase of unlabelled leucine 5 min after the addition of [^{3}H]leucine. The other portion received a chase plus cycloheximide. At the times indicated samples were withdrawn and fractionated. Citrate synthase immunoprecipitated from mitochondria and post-ribosomal supernatant was analysed by SDS–gel electrophoresis. Radioactivities in the M_r 45 000 peak corresponding to citrate synthase were determined. (A) Protein of total cell homogenate; (B) total mitochondrial protein; (C) citrate synthase immunoprecipitated from mitochondria; (D) citrate synthase immunoprecipitated from post-ribosomal supernatant. (•——•) Chase; (o– –o) chase plus cycloheximide.

ported from a variety of organisms [15]. Mitochondrial lysates from cells grown in the presence of [^{35}S]sulphate were treated with antibody against the isolated protein and the immunoprecipitates subjected to SDS electrophoresis. The immunoprecipitates yielded a single labelled band with app. mol. wt 45 000 (fig.1). An identical band could also be obtained from the post-mitochondrial supernatant of



cell homogenates. The latter arises from redistribution of the mitochondrial protein during cell fractionation; this point will be discussed further in relation to fig. 2.

^{35}S-Prelabelled cells were pulsed with [^{3}H]leucine at 8°C for 5 min. The cell suspension was halved; to one half a chase of unlabelled leucine was given while to the other a chase plus cycloheximide was given. The cells were harvested at the intervals shown, ground and fractionated in buffer. Citrate synthase was immunoprecipitated from the mitochondrial fraction and post-ribosomal supernatant. The immunoprecipitates were subjected to SDS–gel electrophoresis, the gels fractionated and radioactivities determined in the slices. The gels all showed a single peak of radioactivity with M_r 45 000. The progress of the ^{3}H/^{35}S ratios of the protein peak was plotted against time and the results are shown in fig.2. The increase in specific activity in total cellular protein is plotted in fig.2A. Addition of chase or chase plus cycloheximide immediately arrested the increase in radioactivity which clearly shows that the chase was effective. It was shown that cycloheximide immediately blocks further translation in this system [2,3]. In contrast the isolated mitochondria (fig.2B) showed an increase in ^{3}H radioactivity following both treatments. This is qualitatively and quantitatively consistent with published data [2]. The chase prevents labelling of intramitochondrial proteins and rules this out as a possible source of increase of the specific radioactivity in the mitochondria.

The ^{3}H/^{35}S ratio of purified citrate synthase shows a strong increase after the chase and after the chase plus cycloheximide. This holds for both the mitochondria and the cytosol fraction. The kinetics suggest a post-translational transfer of citrate synthase into the mitochondria, since cycloheximide did not prevent further appearance of labelled enzyme in the mitochondria. They are not in accord with the cytosolic form of the protein being the precursor of the mitochondrial protein. If such were the case an initially higher ^{3}H/^{35}S ratio with an ensuing decrease would be expected. The existence of an undetected precursor pool of protein must however be considered. The possibility arises that the precursor is very unstable and thus eludes detection or else that the antibody against mature enzyme does not recognize such a precursor.

We investigated the existence of a precursor by immediate immunoprecipitation from extracts of whole cells. Pulse labelling of sulphate-depleted cells which had been prelabelled by [^{3}H]leucine was carried out with [^{35}S]sulphate. This procedure resulted in high specific labelling of cellular protein. Cells sampled at intervals were quickly harvested at 0°C, frozen in liquid nitrogen and extracted. Citrate synthase was immunoprecipitated from a 12 000 × g supernatant. The immunoprecipitates were subjected to SDS electrophoresis. The radioactivity profiles of the gels are shown in fig.3. The earlier samples show the progressive appearance of a ^{35}S-labelled protein peak with an M_r larger than the mature protein. This peak is ultimately supplanted in the last sample by the ^{35}S-label in the mature protein. The same higher molecular form of citrate synthase was detected when cells were pulse labelled with methionine of high specific radioactivity. The amounts of precursor were always very low compared to the authentic protein.

In order to verify the existence of a precursor form of citrate synthase, in vitro translation experiments were performed. In a homologous cell-free read out system [3] [^{35}S]methionine was incorporated for 20 min and citrate synthase immunoprecipitated

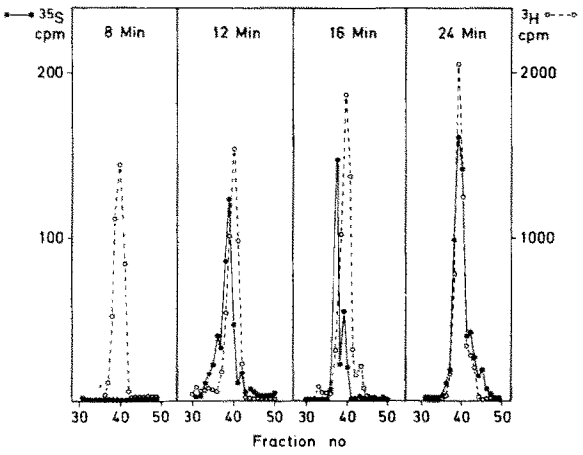


Fig.3. Synthesis of citrate synthase in vivo. Cells prelabelled with [^{3}H]leucine were pulsed at 8°C with [^{35}S]sulphate. Citrate synthase was immunoprecipitated at the intervals shown from cell extracts and subjected to SDS–gel electrophoresis. The gels were fractionated and the distribution of radioactivities determined.

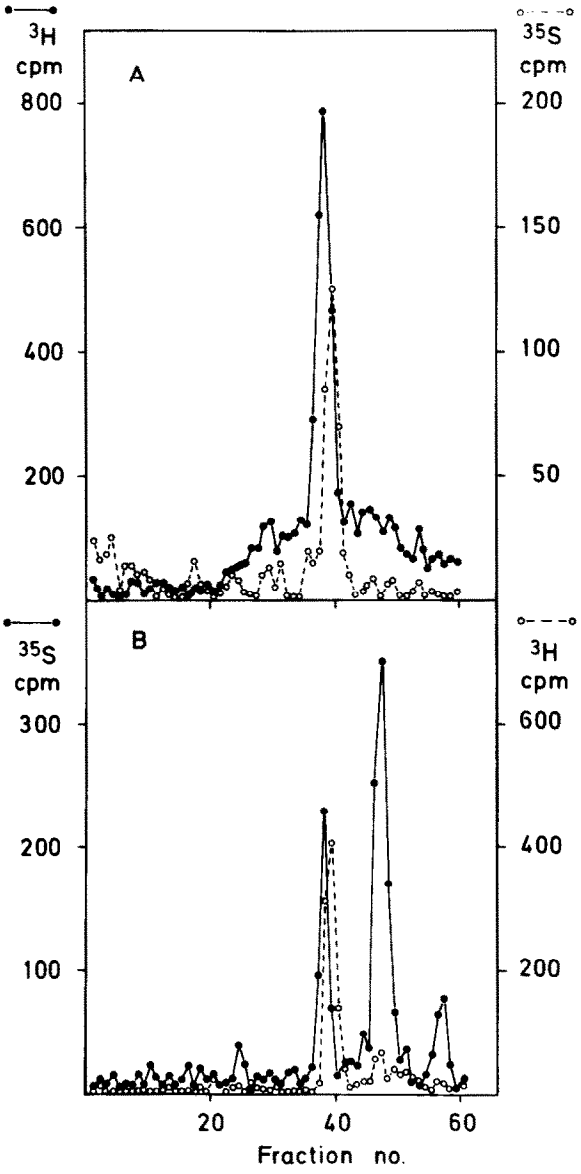


Fig.4. Synthesis of citrate synthase in vitro. (A) A ^{35}S-prelabelled cell free homogenate was labelled with [^{3}H]leucine at 25°C. Following 20 min incubation, citrate synthase was immunoprecipitated from the post-ribosomal supernatant. The immunoprecipitate was subjected to SDS electrophoresis, the gel fractionated and the distribution of radioactivities determined. (B) Poly(A)RNA from *Neurospora* was translated in a rabbit reticulocyte lysate. Following incorporation of [^{35}S]methionine for 60 min citrate synthase was immunoprecipitated from the post-ribosomal supernatant. The immunoprecipitate was subjected to SDS electrophoresis. Authentic [^{3}H]citrate synthase was co-electrophoresed.

from the post-ribosomal supernatant. The immunoprecipitated protein was analysed by vertical SDS–gel electrophoresis and autoradiography. A single protein band was observed moving slightly slower (M_r 47 000) than the co-electrophoresed authentic protein (fig.1). In view of the small size difference between the two protein bands and to guard against artifacts of precipitation or electrophoresis we carried out a parallel translation using a dual labelling system. A cell free homogenate from ^{35}S-labelled cells was incubated with [^{3}H]leucine and citrate synthase from the post-ribosomal supernatant immunoprecipitated and electrophoresed. The gels were fractionated as above. Figure 4A shows the radioactivity profile of the gels. A prominent ^{3}H-labelled protein peak with M_r 47 000 compared to the M_r 45 000 labelled authentic protein is clearly distinguishable.

Poly-A-RNA from Neurospora was translated in a rabbit reticulocyte system and citrate synthase immunoprecipitated from the postribosomal supernatant. Electrophoresis and autoradiography of the immunoprecipitated protein revealed the presence of a protein band with M_r 47 000 (fig.1). Co-electrophoresis with ^{3}H-labelled authentic protein gave the same result (fig.4B). The immunoprecipitates showed a reproducible pattern of lower molecular weight products. We attribute these to proteolysis. Sensitivity of some translation products of reticulocyte lysates has been reported, e.g., apocytochrome *c* from rat could only be obtained undegraded when special means to inhibit endogenous protease activity were employed (T. Morimoto, personal communication). The lability of the precursor form of citrate synthase is emphasised by the fact that in cell free systems the M_r 47 000 form disappeared completely on prolonged incubation. This is in striking contrast to other mitochondrial precursor proteins studied in our laboratory [9,10].

4. Discussion

The results presented show that mitochondrial citrate synthase is made as a larger precursor both in vivo and in vitro. In pulse-labelled cells the first detectable newly synthesized form of citrate synthase is the larger molecular weight one. With time this form becomes undetectable in the presence of large

amounts of the mature form suggesting that the higher molecular form is the precursor of the mature protein. The property of having a larger precursor is not shared by two other mitochondrial proteins studied in *Neurospora,* viz., ADP/ATP translocator protein [9] and cytochrome *c* [10]. Citrate synthase in this respect is similar to a number of other imported mitochondrial proteins [6–8,11]. The kinetics of appearance of citrate synthase in mitochondria in pulse-chase experiments followed by cycloheximide suggest that the import is post-translational and are in agreement with the published data for precursor import into mitochondria [2,3,16,18]. However, the instability of the precursor coupled with the leakage of mature protein from the mitochondria presented a problem in assigning a subcellular localisation to the precursor form. The most probable interpretation of the results would locate the precursor in the cytosol fraction. The data however do not rule out a presence of the precursor in the mitochondria which due to its characteristic lability could be lost during immunoprecipitation. It is pertinent to this point that no clear indication is evident from published data to date precisely where cleavage takes place. Nor has it been shown that cleavage and uptake (in the narrow sense of entry into the mitochondria) are obligatorily linked. It is however clearly shown by the data here presented and that of others that cleavage is post-translational [16]. Schatz and coworkers have demonstrated that the cleavage is performed by mitochondria [16].

The biological role of the additional sequences on mitochondrial precursor proteins remains unclear. It is not known if cleavage is necessary for transfer as some proteins which do not require cleavage are transferred into mitochondria [4,9,10]. Import of a mature dimeric mitochondrial matrix protein has been reported [17]. This latter observation is difficult to reconcile with the view that cleavage of precursor proteins is an intrinsic feature of the uptake mechanism [16]. We envisage that the additional sequence prevents premature assembly in the cytoplasm which would lead to mislocation. A protease inside the mitochondria could trigger the assembly process which in the case of citrate synthase would result in the formation of the dimer.

Acknowledgements

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